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Award Number: DAMD17-01-1-0510

TITLE: Study of RANKL Expression in Metastatic Breast Carcinoma

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REPORT DATE: June 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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REPORT DOCUMENTATION PAGE			Form Approved OMB No. 074-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503				
1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE June 2002	3. REPORT TYPE AND DATES COVERED Annual (1 Jun 01 - 31 May 02)		
4. TITLE AND SUBTITLE Study of RANKL Expression in Metastatic Breast Carcinoma		5. FUNDING NUMBERS DAMD17-01-1-0510		
6. AUTHOR(S) Pardeep Bhatia, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Connecticut Health Center Farmington, Connecticut 06030  E-Mail: <a href="mailto:bhatiapardeep@hotmail.com">bhatiapardeep@hotmail.com</a>		8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER		
11. SUPPLEMENTARY NOTES Report contains color				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words)  Bone is the most common site of metastases by human breast cancer. Most breast cancers form osteolytic metastases, in contrast to tumors such as prostate cancer that form osteosclerotic metastases. Although some evidence suggests that formation of bone metastases by breast cancer cells is mediated by the increased osteoclastogenesis at the target site, a clear controversy exists whether formation of bone metastases is mediated by breast cancer cells directly or by stimulated osteoclasts. We have therefore examined the expression of RANK and RANKL, two proteins importin the bone remodeling signaling pathway, in invasive carcinoma of breast and bone metastases of breast. We observed both RANK and RANKL were upregulated in these breast tumors and metastases. Further, breast tumor cells were directly in contact with the bone without any osteoclasts in the vicinity. We suggest that overexpression of RANK and RANKL in breast cancer cells provides a growth advantage to the breast tumor cells, and the tumor cells appear to be directly responsible for the degradation of bone.				
14. SUBJECT TERMS breast carcinoma, metastasis, RANKL			15. NUMBER OF PAGES 13	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

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## Introduction

Bone is one of the most common target sites for cancer metastasis. Tumors such as breast carcinoma have a predilection for metastasizing to bone to form osteolytic lesions. Two models have been postulated to explain the bone destruction associated with breast cancer metastasis: the osteolysis is mediated either directly by tumor cells, or indirectly by osteoclasts. Cancer cells have been demonstrated to induce osteoclast differentiation and proliferation by several osteotrophic factors such as IL-1, IL-6, LIF, prostaglandin E2, tumor necrosis factor- $\alpha$  and parathyroid hormone related protein (PTHrP) or by direct cell-cell interaction with bone marrow cells (Mundy 1997, Guise, 1997, Guise and Mundy 1998, Thomas et, 1999). Recently, Receptor Activator of NF- $\kappa$ B ligand (RANKL) and its receptor RANK, have been identified and implicated in regulation of bone remodeling (Holfbauer et al., 2001). RANKL is expressed on stromal cells and osteoblasts and is thought to mediate the interaction between these cells and pre-osteoclasts by binding to its receptor RANK which is expressed on the pre-osteoclasts. This interaction leads to osteoclastogenesis and bone resorption (Holfbauer et al., 2001). Thus the RANK and RANKL interaction appears to play a key role in the process of osteoclastogenesis and bone resorption. Expression of RANKL in other tumor types such as prostate cancer has also been reported (Brown et al., 1999). In addition, studies in mouse model system has generated evidence for the involvement of RANKL in metastatic bone destruction. (Guise 1997, Guise and Mundy 1998). Therefore my hypothesis was that in breast carcinoma expression of RANKL might correlate with bone metastasis and osteolysis.

### Specific Aims:

- To examine different histological forms of breast carcinoma as well as metastatic breast cell lines for expression of RANKL and correlate this expression with phenotype.
- If RANKL expression is detected, to test the ability of anti-RANKL antibodies to inhibit the formation of osteolytic lesions in a model system

## Body

It is well established that breast cancers have the capability to establish and grow as metastases in bone, however, the mechanism underlying their ability to induce osteolysis remains uncertain. *In vitro* studies have demonstrated that breast cancer cells alone have the capacity to degrade the bone matrix, although these lesions of bone or dentine slices are not of the magnitude of those resulting from osteoclast-mediated bone destruction (Eilon and Mundy, 1978). Studies have also shown that PTHrP is expressed by the metastatic breast cancer cells and is a critical component in the mechanism of breast cancer metastases to bone (Boyce et al., 1999; Chirgwin and Guise, 2000; de la Mata et al., 1995; Guise, 1997; Guise and Mundy, 1996; Guise et al., 1996; Guise et al., 1993; Henderson et al., 2001; Kohno et al., 1994; Thomas et al., 1999; Uy et al., 1995; Uy et al., 1997; Yoshida et al., 2000). Co-culture experiments have shown that breast cancer cells can produce both PTHrP and M-CSF which induce RANKL mRNA levels and inhibit OPG mRNA levels in osteoblasts *in vitro* (Mancino et al., 2001; Thomas et al., 1999).

We first investigated the expression of RANKL and its receptor RANK on an array of 60 primary and 10 bone metastatic breast tumors by immunohistochemistry using anti-RANKL and anti-RANK antibodies. These arrays of infiltrating ductal carcinoma (IDC) were obtained from Imgenex, (San Diego, CA) while bone metastatic tumors were obtained from the department of Pathology, University of Connecticut Health Center. Samples were prepared from paraffin embedded archival samples. They were cut using microtome and spread on polylysine coated slides. Tumors were stained using the standard protocol (Herrington and McGee, 1992). Briefly slides were deparaffinized with xylene, dehydrated in alcohol and treated with 4N HCl at 37 C for 10 min. to retrieve the antigen. Samples were washed in distilled water and stained with anti-RANKL and anti-RANK antibodies using Histostain-SP kit (Zymed, South San Francisco, CA). Samples were mounted and photographed under the microscope. Same batch of tumors was also stained with H&E. We encountered a major problem in the antigen retrieving step. However, we have now been able to detect staining of both RANKL and RANK antigens. We observed that the breast cancer tumor cells directly invade the bone in the osteolytic lesions and no osteoclasts are present in the zone of lysis. (Fig1) We also observed that both primary Infiltrating Ductal carcinoma (IDC) as well as metastatic tumors overexpress RANKL and RANK in comparison to non-neoplastic breast (Fig 1-5 ).

Since we observed that normal breast epithelial cells as well as in primary and metastatic breast tumors express RANK on their cell surface, it is possible that during the formation of bony metastases, the metastatic tumor cells would express PTHrP and M-CSF which would stimulate adjacent osteoblasts to express RANKL and suppress OPG expression. This increased expression of RANKL on the surface of the osteoblasts, could interact with the RANK expressed on the surface of the breast cancer cells and stimulate the breast tumor cells to initiate osteolysis (Hunt et al., 2001). This destruction of the bone would release a variety of cellular growth factors such as TGF- $\beta$  (Chirgwin and Guise, 2000; Yin et al., 1999) that could stimulate further growth by the breast cancer cells, leading to increased osteolysis and thus a stimulatory growth loop. Thus while it is likely that breast cancer tumor cells can act to stimulate osteoblasts to recruit osteoclasts

to an osteolytic lesion (Figure 6, Model 1), it is also possible that the breast cancer cells can themselves generate osteolytic lesions in the absence of osteoclasts by a direct interaction between the osteoblasts and the tumor cells (Figure 6, Model 2). In order to test this hypothesis, we are planning to study the expression of RANKL and RANK on human breast cancer cell lines. If we detect this expression, we intend to continue with our specific aim 2 and investigate whether breast tumor cells are also capable of in vitro bone resorption. If it does occur, we would like to further investigate whether anti-RANK or anti-RANKL antibodies could inhibit bone resorption by tumor cells.

#### **KEY RESEARCH ACCOMPLISHMENTS:**

- I found that breast cancer cells may directly invade the bone resulting in osteolytic lesions of bone metastasis
- I demonstrate that Receptor Activator of NF- $\kappa$ B ligand (RANKL) and its receptor RANK are expressed in primary as well as in bone metastatic tumors of breast carcinoma.
- I discovered that both RANK and RANKL are overexpressed in breast cancer cells of bony metastasis.
- This research supports a model by which formation of osteolytic lesions of bone metastasis by breast tumor cells may be due to the direct interaction of tumor cells which overexpress RANK with the stromal cells which express RANKL.

#### **REPORTABLE OUTCOMES:**

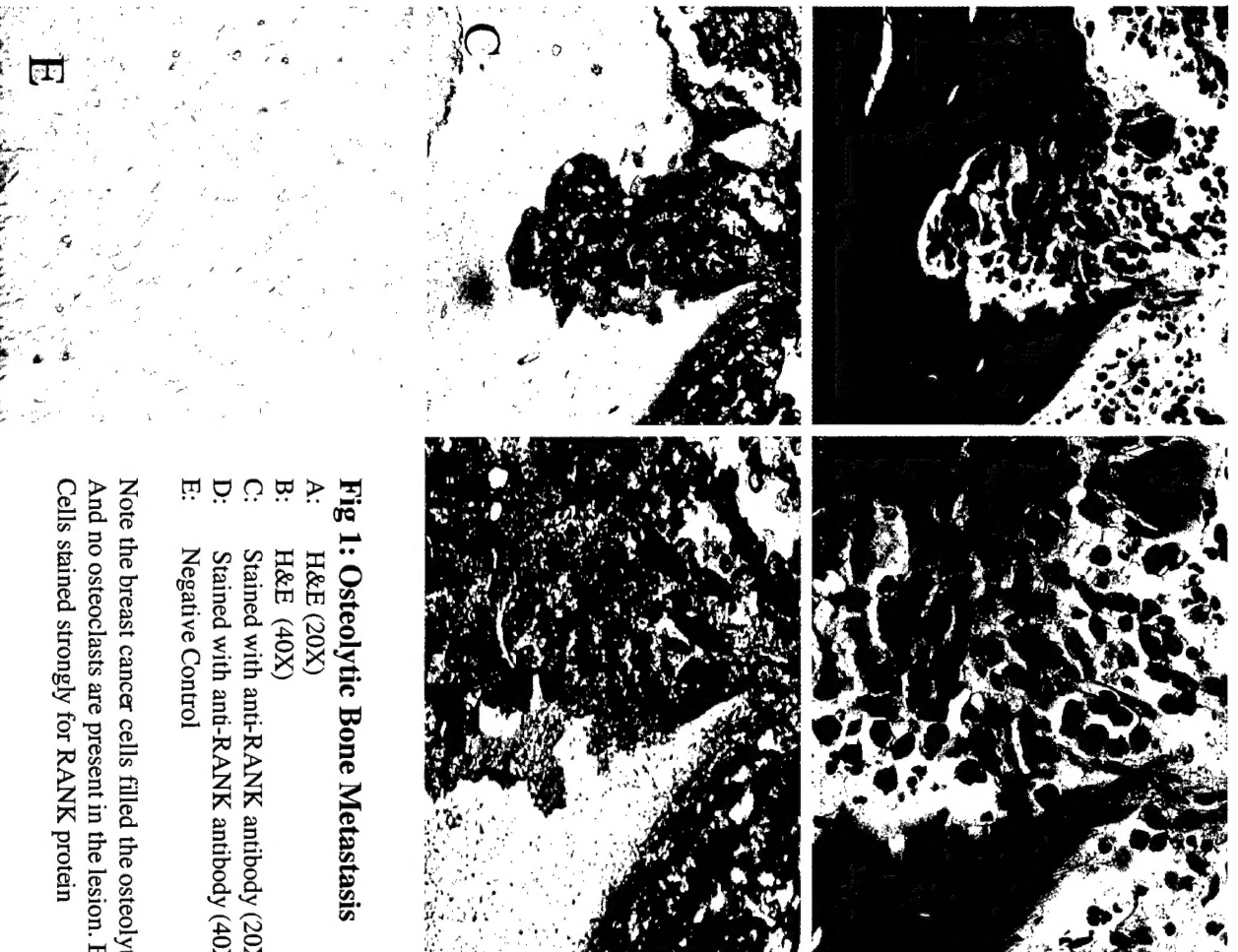
The data reported here was submitted as an abstract and presented as a poster at the Third North American Symposium on Skeletal Complications of Malignancy which took place on April 25-27, 2002 at NIH, Bethesda, Maryland. This abstract will be published in the journal Cancer Research supplement

#### **CONCLUSION:**

Breast cancer cells have the capability to establish and grow as metastasis in bone, however, the mechanism underlying their osteolysis is not understood. A controversy exists whether tumor cells are capable of osteolysis by themselves or is this mechanism mediated by osteoclasts. In the present investigation we have observed that breast cancer cells in bone metastasis overexpress both RANKL and RANK. It is therefore possible that the interaction between RANK on the tumor cells and RANKL on the adjacent osteoblasts/stromal cells could lead to the formation of osteolytic lesions in bone. This may be an alternative mechanism to osteoclastic mediated bone resorption. The implication of this study is that, overexpressed RANK could be a drug target to inhibit bone metastasis by breast tumor cells. It is recommended that further studies are conducted in the animal model systems of breast cancer metastasis using anti-RANK antibodies to inhibit bone metastasis.

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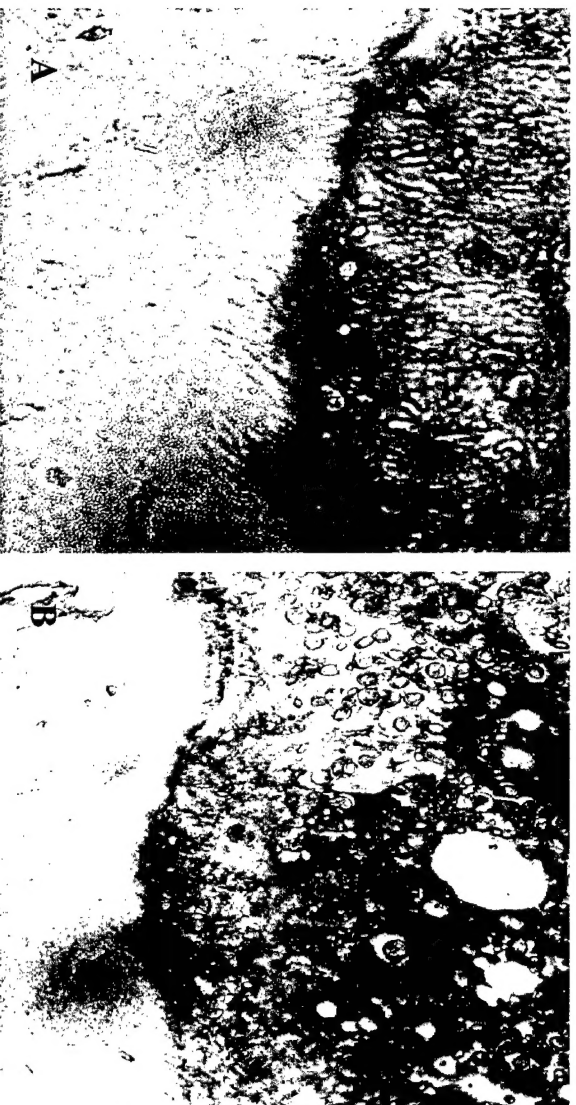
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**Fig 1: Osteolytic Bone Metastasis**

- A: H&E (20X)
- B: H&E (40X)
- C: Stained with anti-RANK antibody (20X)
- D: Stained with anti-RANK antibody (40X)
- E: Negative Control

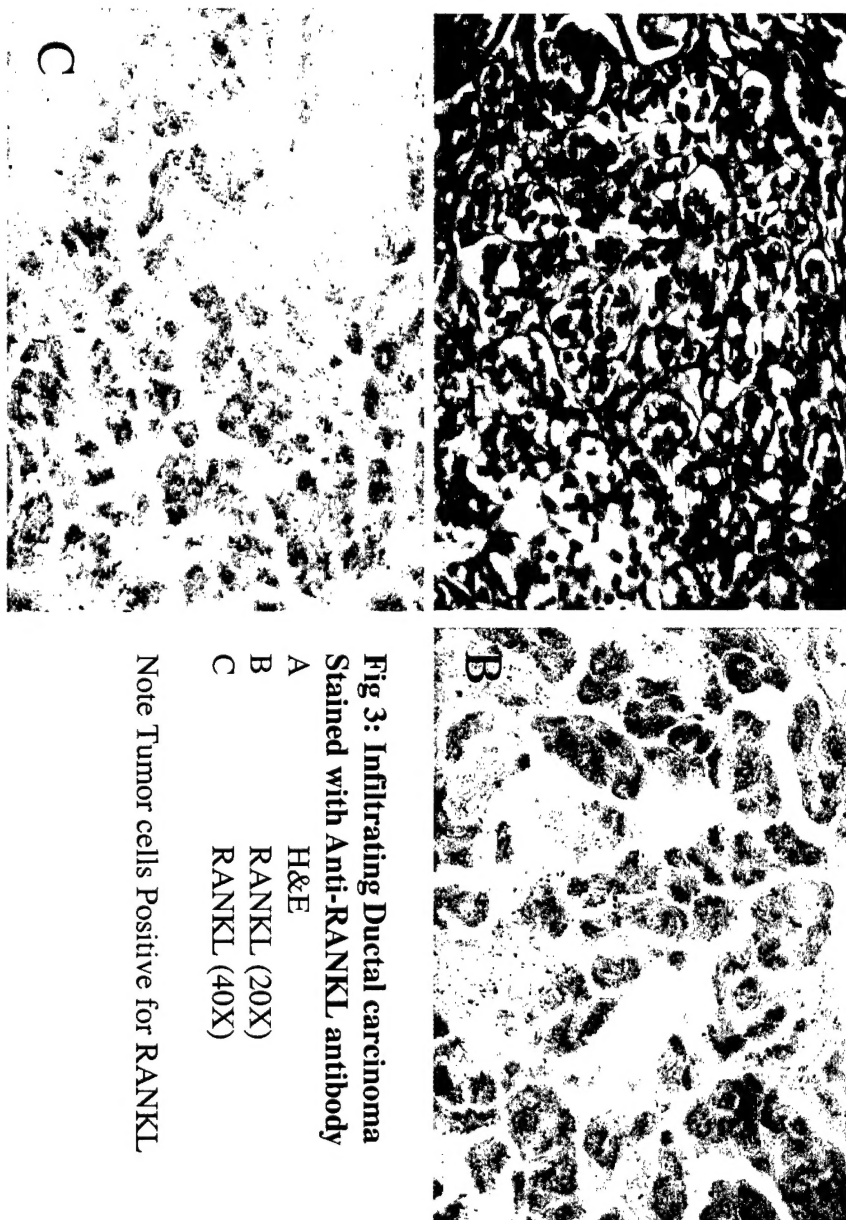
Note the breast cancer cells filled the osteolytic lesions  
And no osteoclasts are present in the lesion. Breast cancer  
Cells stained strongly for RANK protein



**Fig 2: Osteolytic bone metastasis**  
A: RANKL (20X)  
B: RANKL (40X)

Osteoblasts cells stain strongly for RANKL.  
While the tumor cells although still positive  
For RANKL stain less

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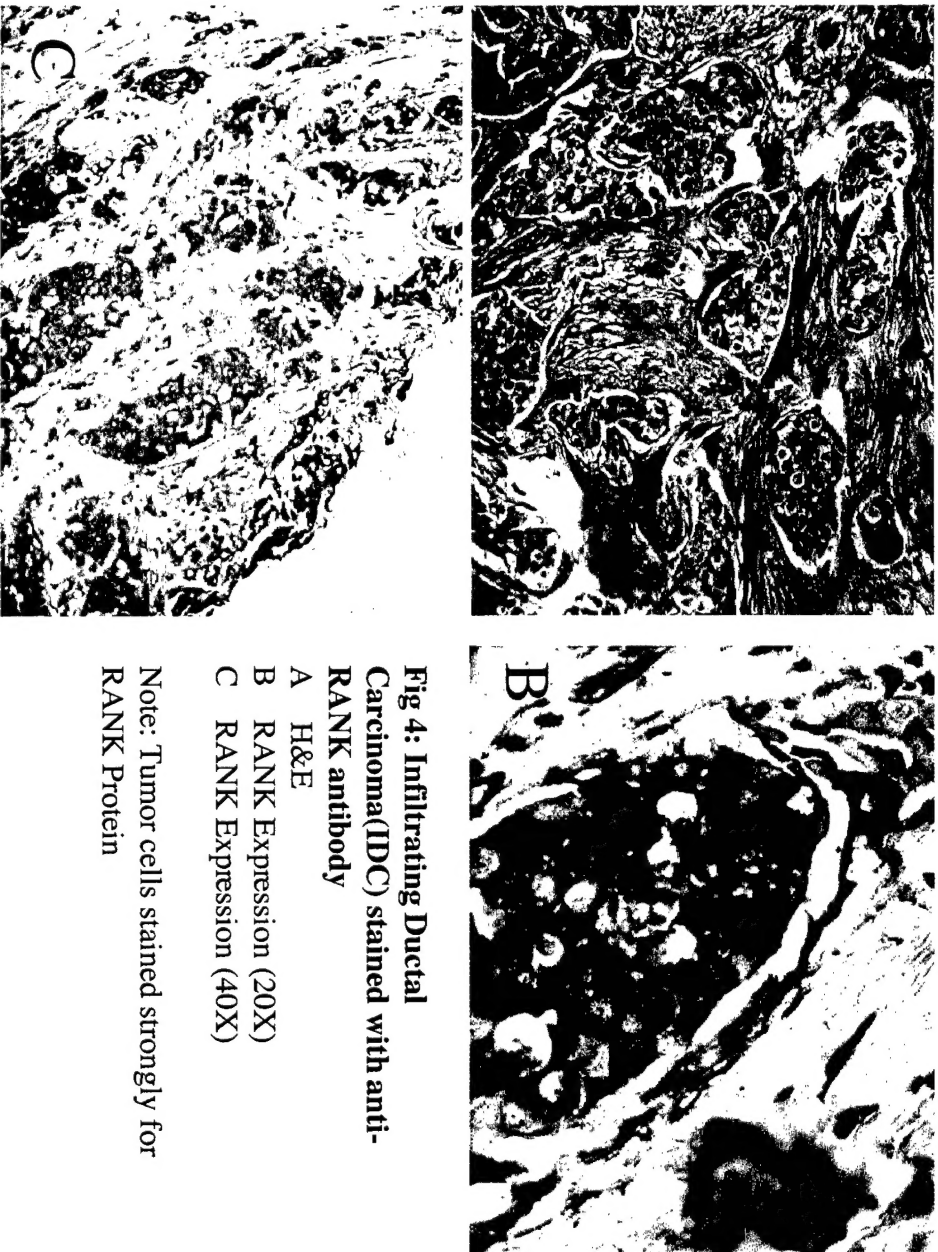


**Fig 3: Infiltrating Ductal carcinoma  
Stained with Anti-RANKL antibody**

- A H&E
- B RANKL (20X)
- C RANKL (40X)

Note Tumor cells Positive for RANKL

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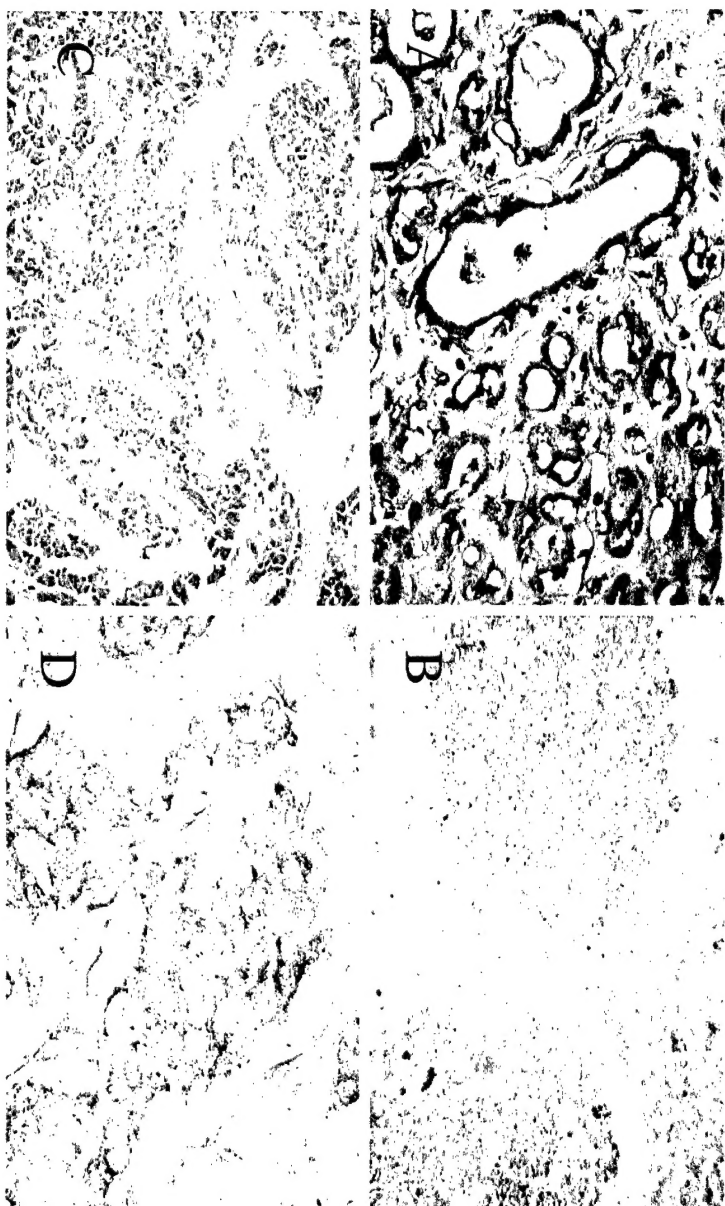


**Fig 4: Infiltrating Ductal  
Carcinoma(IDC) stained with anti-  
RANK antibody**

- A H&E**
- B RANK Expression (20X)**
- C RANK Expression (40X)**

**Note: Tumor cells stained strongly for  
RANK Protein**

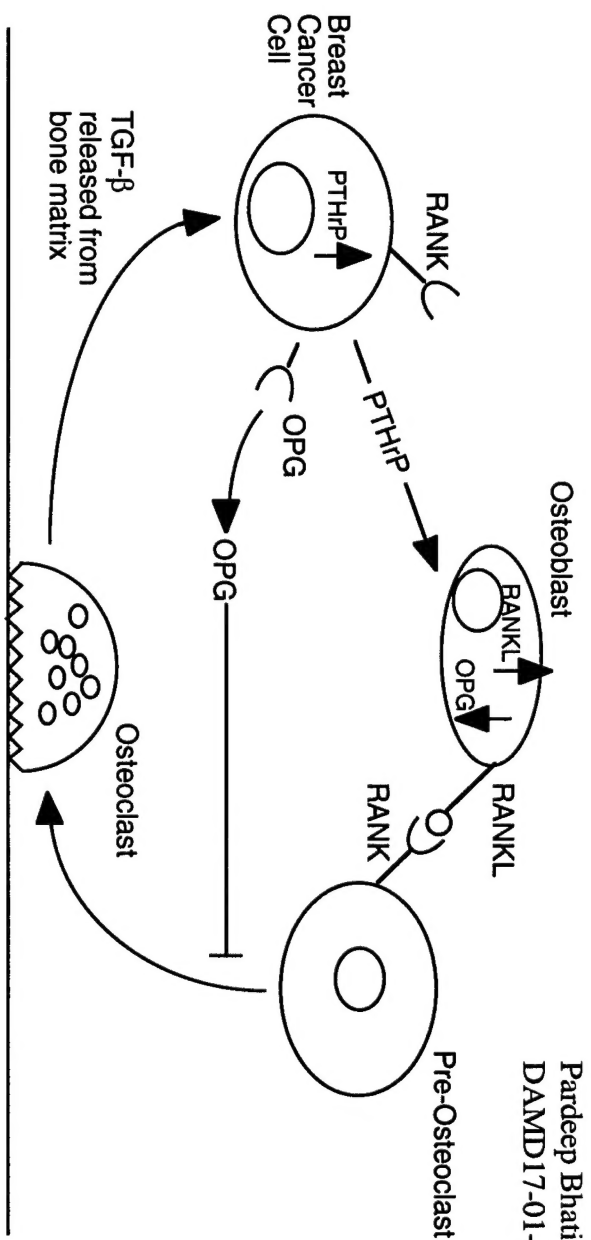
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**Figure 5. Non-neoplastic Breast  
Stained with Anti-RANK and Anti-**

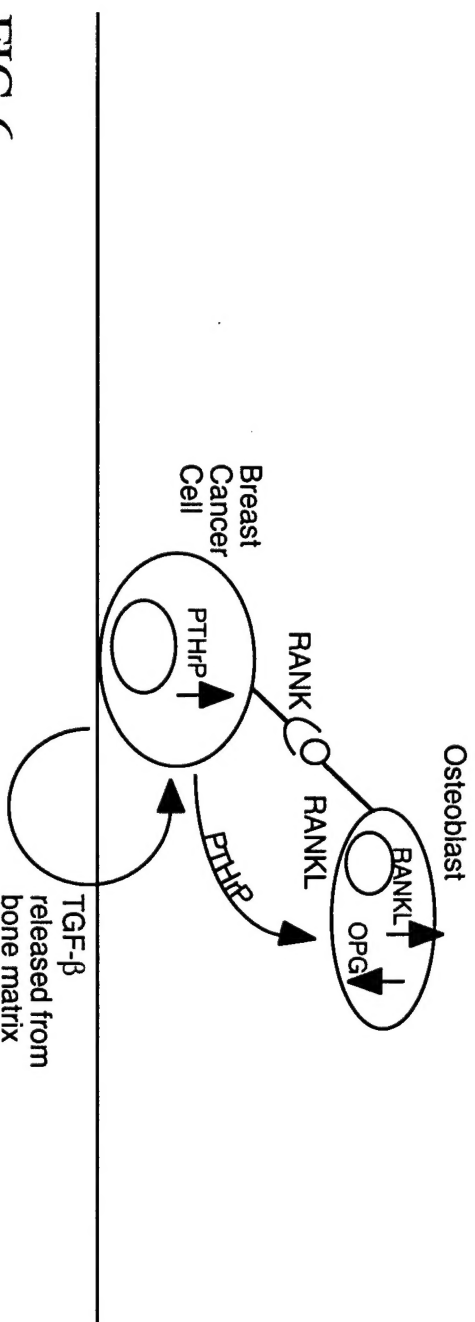
**RANKL Antibody**

- A RANK (20X)
- B RANKL (20X)
- C RANK (40X)
- D RANKL (40X)
- E Negative Control



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Model 1:  
Breast cancer cells secrete  
PTHrP that stimulate  
osteoblasts that in turn recruit  
osteoclasts to form osteolytic  
lesions



Model 2:  
Breast cancer cells secrete PTHrP  
that stimulate osteoblasts that then  
stimulate the breast cancer cells to  
initiate osteolysis directly

FIG 6